Single real-time RT-PCR assay for the detection and quantification of genetically diverse HIV-1, SIVcpz and SIVgor viruses

Running title: (< 54 char) Quantification assay for HIV-1/SIVcpz/SIVgor viruses

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Abstract word count: 249
Text word count: 6140
Abstract

Although antiretroviral treatment availability has improved, the virological monitoring of patients remains largely uneven across regions. In addition, viral quantification tests are suffering from HIV-1 genetic diversity, fueled by the emergence of new recombinants and of lentiviruses from non-human primates. Here, we developed a real-time RT-PCR assay, relatively inexpensive, able to detect and quantify all HIV-1 circulating forms and their precursors, SIVcpz/SIVgor. Primers and a probe were designed to detect all variants of the HIV-1/SIVcpz/SIVgor lineage. HIV-1 M plasma (n=190; 1.68-7.78 log_{10} copies/ml) representing eight subtypes, nine CRFs, and 21 URFs were tested. The mean PCR efficiency was 99% with low coefficients of intra-/inter-assay variation (<5%), and a limit of quantification <2.50 log_{10} copies/ml, with a 200 µl plasma volume. On the studied range, the specificity and the analytical sensitivity were 100% and 97.4%, respectively. Viral loads were highly correlated (r=0.95, p<0.0001) with the reference method (Generic HIV assay, Biocentric) and had no systematic difference, irrespective of genotype. Furthermore, 22 HIV-1 O plasmas were screened and were better quantified compared to the gold-standard RealTime HIV-1 assay (Abbott); including four samples that were only quantified by our assay. Finally, we could quantify SIVcpzPtt/SIVcpzPts from chimpanzee plasma (n=5) and amplify SIVcpz/SIVgor from feces. Thus, the newly developed real-time RT-PCR assay detects and quantifies strains from the HIV-1/SIVcpz/SIVgor lineage, including a wide diversity of group M strains and HIV-1 O. It can therefore be useful in geographical areas of high HIV diversity and at risk for emergence of new HIV variants.
Background

In 2009, about 5.2 million people in low- and middle-income countries were receiving antiretroviral therapy (ART) (UNAIDS, 2010). Programs to scale-up ART in resource-limited countries have increased the number of people receiving treatment. Nevertheless, viral load (VL) monitoring for patients on ART or for early viral detection in exposed children is only rarely available in resource-limited settings. Scale-up of laboratory monitoring such as VL measurement in low-income countries is a priority, and it has been defined as a recommendation by UNAIDS in 2010 to improve the efficiency and quality of HIV antiretroviral treatment and care.

Today, different viral load assays are available and use different techniques of molecular biology such as real-time RT-PCR (reverse transcription polymerase chain reaction), NASBA (Nucleic acid sequence based amplification), or bDNA (branched-chain DNA signal amplification) (reviewed in (1)). If HIV quantification assays are rarely present or only in reference laboratories of resource-limited countries, it is mainly due to their high cost which can reach 50 to 100 dollars per sample (i.e. higher than the cost of ART) and their need for specific instruments and trained staff (2). Two cheaper alternatives to classical molecular based methods, heat-dissociated HIV-1 p24 antigen ELISA (3, 4) and Cavidi ExaVir the reverse transcriptase activity assay (5, 6), have been proposed, but have shown inconclusive results for the first and need further improvement and evaluation for the second (7-9). Dipstick and chip technologies are still in development (2, 10, 11). Therefore, recent ‘in-house’ or generic real-time RT-PCR assays have shown diverse advantages and are relatively inexpensive (the real-time technology is widely used in HIV and other viral infection detections) (12-14).

The high genetic diversity of HIV-1 is a major challenge for the development of efficient and sensitive quantification assays (1, 2, 14, 15). All commercial quantitative tests were primarily
designed on subtype B viruses and, even if they are continuously optimized to detect a broad range of variants, they still do not quantify effectively all circulating strains (1, 16-18). This drawback is particularly an issue in sub-Saharan African areas where non-B strains predominate, and where many and highly diverse HIV-1 variants co-circulate. Due to globalization, this heterogeneity can also be found in different geographic regions in which the common viral load assays may not be able to detect these ‘unusual’ strains (19-21). Furthermore, highly divergent viruses, such as HIV-1 groups O, N, and P, which also circulate and have a clinical course similar to HIV-1 group M, need appropriate monitoring tools that are not often available (22, 23). Thus, HIV diversity and molecular epidemiology still impacts on the management and monitoring of HIV infected patients. Some recent generic or ‘in-house’ tests have designed their assay to target a wide variety of HIV-1 strains and could validate their technique on multiple samples from different geographic areas (reviewed in (24)). However, the main limit of the majority of these tests is that their designed primers and probe have numerous mismatches with HIV-1 groups O, N, and P (alignment with HIV-1 sequences from the HIV.lanl database, data not shown), implying the possible undetection or under-quantification of these circulating strains (25). On the other hand, a real-time PCR assay developed by Gueudin and colleagues specifically quantifies HIV-1 group O, but does not detect HIV-1 group M strains (22, 26).

To date, no viral load assay has been designed to possibly detect new zoonotic SIV/HIV viruses emerging from SIVs naturally infecting chimpanzees and gorillas, such as HIV-1 group P identified in 2009 from SIVgor (27). Furthermore, recent studies have shown that SIVcpz, ancestors of HIV-1, can be pathogenic for their natural host (28, 29) and we showed previously how difficult it can be to follow such viral infection in plasma and fecal samples. To monitor SIV infection in naturally infected apes, a new tool should be available to detect SIV RNA in fecal samples and to quantify SIV viral load in plasma over the course of infection.
Here, our goal was to design a new RT-qPCR assay, relatively inexpensive and at least equal to generic or ‘in-house’ tests regarding technical characteristics and performance, but with the capacity to virtually detect and quantify all HIV-1 circulating strains. Furthermore, there is still a risk for SIV emergence from infected apes to humans. It will then be important to be able to detect with this same test any hypothetic new emerging SIVcpz or SIVgor viruses in the human population. Finally, a detection and quantification test would be useful to monitor SIV infection in great apes from both fecal and plasma samples, to understand better the course of SIVcpz and SIVgor infections in their natural hosts, reservoir of the ancestors of HIV-1. Thus, our aim was to design a single quantitative viral load assay satisfying these different goals.

**Materials and Methods**

**HIV negative plasma samples**

For negative control and specificity determination, 72 HIV negative plasma samples from patients attending a hospital in Yaoundé, Cameroon, for a HIV test were available. The negativity of these samples was determined by diverse HIV serological tests (ICE HIV-1.0.2 (Murex Biotech Limited, Dartford, UK), Wellcozyme HIV Recombinant (Murex), Determine HIV-1/2 (Abbott Laboratories, Tokyo, Japan)). These samples were processed similarly as the HIV positive samples.

**HIV-1 group M plasma samples**

A total of 190 HIV-1 group M RNA extracts from plasma samples were available for viral quantification (Table 1). All of them were previously analytically detected with the Generic HIV-1 viral load Biocentric kit for clinical studies and conserved at -80°C. Their VL ranged between 1.68 and 7.78 log_{10} copies/ml, with 12 out the 190 plasma samples being analytically detected
(PCR amplification) but under the threshold of quantification determined by Biocentric (Biocentric quantification threshold, 2.5 log_{10} copies/ml) (30). The remaining 178 plasma samples were clinically positives as quantified by the Biocentric technique (above 2.5 log_{10} copies/ml).

For 167/190 HIV-1 group M positive plasma samples that had a VL superior to 3 log_{10} copies/ml, a region of approximately 1,865 bp in pol (protease and reverse transcriptase regions) was amplified and sequenced as previously described, primarily to determine the drug resistance profile and genotypes for previous studies (31-33) (Peeters M, unpublished results). The different subtypes and CRFs (Circulating recombination forms) are shown in Table 1. Our evaluation included plasma samples from four different countries of Africa with different HIV-1 subtypes/CRFs distribution: 18 from Burundi and 54 from Togo with a relatively low genotypic heterogeneity (mainly subtype C and CRF02, respectively), 65 from Cameroon and 39 from DRC (Democratic Republic of Congo) with highly diverse subtypes and CRFs. We also included 14 plasma samples with HIV-1 subtype B strains from the hospital of Montpellier, France (34). The panel covered the heterogeneity of subtypes and CRFs of HIV-1 group M circulating strains: all subtypes, with the exception of subtype K, were represented, major CRFs were also present, and 21 URFs (Unique recombinant forms) were included (19). This panel also comprised 23 samples from which genotyping was not possible or not performed due to their low viral loads (< 3 log_{10} copies/ml), but they were kept in the study since we wanted to test our assay on samples with very low viral loads too.

HIV-1 group O plasma samples

We tested 31 HIV-1 group O samples with our new RT-qPCR assay. All samples were identified as HIV-1 O by a previously reported in-house Elisa using V3-loop peptides from HIV-1 groups M, N, and O (35). Twenty-two samples were plasma samples from Cameroon conserved...
at -80°C and previously quantified by Abbott RealTime™ test (Abbott Molecular Inc., Les Plaines, IL) in Yaoundé, Cameroon. For the nine remaining samples, only DNA extracts, with HIV-1 group O sequence confirmation, conserved at -20°C, were available for detection, but they allowed us to further test our assay on various HIV-1 group O strains. For HIV-1 group O positive samples, a small region in env (gp41) of approximately 450 bp and/or a region in pol (reverse transcriptase) of approximately 1,800 bp were amplified and sequenced when enough material was available (14/31 samples). Sequences were aligned with ClustalW (36) and phylogenies were performed using PhyML (37) with the GTR model with four gamma distributed rate categories. The group O strains sequenced and tested in this study covered the HIV-1 group O genetic diversity (Figure 1A).

**SIVcpz plasma samples from chimpanzees**

We tested plasma samples from three previously described SIVcpz infected chimpanzees and one non-infected chimpanzee as a negative control. Two SIVcpz+ chimpanzees (Gab2 and Ch-Go) are from the Pan troglodytes troglodytes subspecies and were infected with SIVcpzPttGab2 (38, 39) and SIVcpzPttCam155 (28), respectively. These strains cluster in the SIVcpzPtt/HIV-1M/HIV-1N lineage close to other SIVcpzPtt infecting chimpanzees from Cameroon and Gabon (black arrows in Figure 1B). Ch-No, the third SIVcpz positive chimpanzee, is from the P.t.schweinfurthii subspecies and was infected with SIVcpzPts-ant that clusters in the monophyletic lineage of SIVcpzPts strains, out of the SIVcpzPtt/SIVgor/HIV-1 lineage (40, 41) (black arrow in Figure 1B). Sequential plasma samples were available for Ch-Go (two time points seven years apart) and for Ch-No (four time points between October 1989 and January 1991).

**HIV/SIV RNA extraction from plasma samples**
HIV and SIV RNA were extracted from 200 µl of plasma, conserved at -80°C, using QIAamp Viral RNA Mini kit (Qiagen, Courtabeuf, France) and eluted with 60 µl of elution buffer. Standards and the reproductive control, provided by Generic HIV-1 viral load Biocentric kit (Biocentric, Bandol, France), were inactivated culture supernatants of HIV-1 subtype B and were extracted with the same protocol.

**Fecal samples from wild living chimpanzees and gorillas infected with SIV**

We tested 78 fecal samples, conserved in RNAlater™, from chimpanzees (n=24) and gorillas (n=54) from Cameroon previously described to have HIV-1 cross-reactive antibodies (42, 43). In these previous studies, we were able to amplify and sequence fragments in pol and/or gp41 viral regions from five chimpanzee samples (5/24) and from fifteen gorilla samples (15/54), after two to ten independent RNA extractions and subsequent RT-PCR attempts. These strains represented the genetic diversity of SIVcpzPtt and SIVgor viruses (white arrows in Figure 1B).

Here, we extracted total RNA from 1.5 ml of each ape’s fecal sample, using the NucliSens Magnetic Extraction kit (Biomérieux, Craponne, France) as previously described (43), to obtain a final RNA extract volume of 50 µl.

**Development of a real-time RT-qPCR assay for detection and quantification of viral strains from the HIV-1/SIVcpz/SIVgor lineage**

We designed the primers and probe using an alignment of sequences from various HIV-1 strains from all four groups (M, N, O, and P), SIVcpzPtt and SIVcpzPts, and SIVgor viruses. The LTR region was firstly explored according to previous studies, which choose this region because of its low variability across HIV-1 strains (26, 44-46). The alignment we made with more divergent viruses (SIVcpz/SIVgor) and with all sequences available from divergent HIV-1 groups (N, O, P), including some not available at the time of the previously reported assays in the LTR region, highlighted the numerous mismatches of previously described primers and probes. Thus,
we designed new ones that were set to amplify HIV-1 groups M, N, O, and P, as well as SIVcpzPtt, SIVcpzPts, and SIVgor viruses. The reverse primer (HXB2 position 622-642, 5’-AAAATCTCTAGCAGTGCC-3’) was similar to a previously described primer (30), and the forward primer (HXB2 position 523-539, 5’-SSCTCAATAAGCTTGC-3’) had a similar position and length than previously described (46) but we changed two nucleotides at the 5’end to match all divergent strains. These primers, matching all aligned sequences with 100 % homology, amplified a small fragment of 120 bp. The new probe (HXB2 position 588-603, 5’-CTAGAGATCCCTCAGA-3’) was designed in a similar position than previously reported (45) but was 10 bp shorter and had different characteristics, it was a reverse internal TaqMan probe carrying a 5’ FAM reporter and a 3’ minor groove binding – non-fluorescent quencher (Applied Biosystems, Foster City, CA). It can be noted that one mismatched nucleotide residue at the 3’-end was observed for three SIVcpzPts strains (SIVcpzPtsTAN1, 2, 3).

We performed all runs in a 20 µl reaction volume containing 10 µl of RNA extract, the primers and the probe at 500 nM, 1X of TaqMan Fast Virus 1-step Master Mix (Applied Biosystems) and RNase-free water to the final volume. Thermal cycling conditions were as follows: reverse transcription at 50°C for 5 min, RT inactivation and initial denaturation at 95°C for 20 sec, and amplification with 50 cycles at 95°C for 3 sec and 58°C for 30 sec (total duration ~ 70 min). Cycling and data acquisition were carried out using the 7500 Real Time PCR system (Applied Biosystems). We used five standards from the Optiquant™ HIV-1 RNA Quantification Panel (2.78, 3.78, 4.78, 5.78, 6.78 log_{10} copies/ml) and the Optiquant™ HIV-1 RNA low-positive control (3.78 log_{10} copies/ml) (Biocentric). We assessed the maximum lower-limit at which a sample can be correctly quantified by diluting the 3.78 log_{10} copies/ml standard to two lower concentrations (2.50 and 1.78 log_{10} copies/ml) and tested them in eight replicates.
**RT-qPCR reference techniques**

The Generic HIV-1 viral load Biocentric assay was used as a reference for group M detection and quantification, following the manufacturer’s instructions. This Biocentric assay was previously validated as compared to Versant bDNA HIV RNA kit v3.0 (Siemens Healthcare Diagnostics Inc., Deerfield, IL) and Amplicor HIV-1 Monitor standard RT-PCR assay v1.5 (Roche Molecular Systems, Pleasanton, CA) (30, 46) and has the capacity to detect a wide diversity of HIV-1 group M subtypes and CRFs. Using 200 µl of plasma, the threshold of the Biocentric assay was set at 2.50 log<sub>10</sub> copies/ml. The total duration of the amplification was ~120-140 min. Cycling and data acquisition were carried out using the ABI Prism 7000 Sequence Detection System or the 7500 Real Time PCR system (Applied Biosystems). The Generic Biocentric technique was performed in the IRD laboratory in Montpellier, France. The Abbott m2000rt RealTime™ HIV-1 assay (Abbott Molecular Inc., Des Plaines, IL) was used as a reference for group O detection and quantification, since it was previously validated for HIV-1 group O sample quantification (18, 23). This technique was performed in the IMPM/IRD laboratory in Yaoundé, Cameroon, according to the manufacturer’s instructions.

**Statistical analyses**

The STATA software package version 10.1 (Stata Corp., College Station, Texas) was used for all statistical analyses described. The standards and the low-positive control were tested in ten independent runs to determine the reproducibility, the linearity, and the between-run variability of our RT-qPCR technique. We assessed within-run variability by testing six different samples (five standards and the low-reproductive control) in eight replicates in the same run. The specificity of our assay was calculated as the number of negative samples out of the total number of tested samples from uninfected individuals. The analytical sensitivity for HIV-1 M RNA was calculated as the number of samples detected with our technique divided by the number of samples detected.
with the Biocentric generic assay, including samples under the quantification threshold. Correlation between results from Generic Biocentric test and our new RT-qPCR assay were measured by a Pearson correlation coefficient and by a Spearman rank correlation coefficient for results from each country. We generated a Bland-Altman difference plot for bias and agreement measurements, including limits of agreement (47).

Results

Reproducibility, and variations between and within runs.

The inter-assay reproducibility of the standard curve with our new RT-qPCR method was assessed on ten independent assays. In all cases, there was a strong linear correlation between the cycle threshold values found in each experiment and the viral load (log_{10} copies/ml) with a median correlation coefficient of 1.00 (range, 0.99 to 1.00). The mean slope of the standard curve was -3.33 (range, -3.44 to -3.15), corresponding to a mean amplification efficiency of 99.2\%.

The standard with the lowest concentration (2.78 log_{10} copies/ml) was always detected and amplified. The diluted sample at 2.50 log_{10} copies/ml was always detected and quantified with a low coefficient of variation (inferior to 15\%), whereas the diluted sample at 1.78 log_{10} copies/ml was detected in six out of eight replicates. Thus, our RT-qPCR assay has a quantification threshold inferior to 2.50 log_{10} copies/ml using an input volume of 200 \mu l of plasma (limit included in the 1.78-2.50 log_{10} copies/ml interval). The low-positive control at 3.78 log_{10} copies/ml was added to each test and was used to further assess the reproducibility and determine the between-run variation. The mean value of this positive control was 3.83 log_{10} copies/ml (SD, +/- 0.19) with a coefficient of variation of 4.8\%. These data are highly similar to what has been
determined for the Generic HIV viral load Biocentric assay, confirming a good inter-run reproducibility.

To assess the within-run variation, the standards (n=5) and the low-positive control were each replicated eight times in the same experiment. The low-positive control and the standards were always detected and correctly quantified with a mean coefficient of variation of 4.0 % (SD, +/- 0.2).

The analyses of HIV-1 group M and HIV negative samples show that the assay has a good sensitivity and specificity.

For the analytical evaluation, a total of 190 HIV-1 group M positive plasma samples were detected by Generic HIV viral load Biocentric assay and tested with the new RT-qPCR test (VL range, 1.68 to 7.78 log_{10} copies/ml). Out of them, 185 plasma samples were effectively detected with our technique (VL range, 2.14 to 8.07 log_{10} copies/ml). The analytical sensitivity of our assay could be estimated at 97.4% (CI95, 94.0 to 99.1%). Five samples, with a Biocentric viral load between 2.18 and 3.04 log_{10} copies/ml, were not detected with our assay, including three under the Biocentric quantification threshold (2.5 log_{10} copies/ml). Two samples had a viral load superior to the quantification threshold: one from Cameroon (VL(Biocentric), 3.04 log_{10} copies/ml) from which no genotype could be obtained despite two amplification attempts in the conserved pol region, and one from DRC (VL, 2.94 log_{10} copies/ml) with no genotyping available. The specificity of the test was assessed with 72 HIV negative samples from Cameroon. All samples yielded negative results with our test. Thus, the specificity of the assay was 100% (CI95, 95.9 to 100%).

Good correlation between the new test and the reference Biocentric assay for the quantification of HIV-1 group M, irrespective of the genotype.
Biocentric HIV viral load results and new RT-qPCR assay viral load measures were both available for 185 HIV-1 group M plasma samples. As shown in Figure 2A, an excellent correlation was found between the results of both assays (Pearson correlation coefficient r=0.95; p<0.0001). Considering the quantification threshold of 2.5 log_{10} copies/ml, the Biocentric assay and our RT-qPCR test quantified 178 and 179 samples above this limit, respectively. Three samples were under the threshold with both techniques, while four and three samples were under the threshold with Biocentric and our assay, respectively (Figure 2A). We determined the agreement between the two assays by the Bland-Altman difference plot (Figure 2B) (47). The mean viral load difference between the two tests was of 0.02 log_{10} copies/ml and was not significantly different from 0 (p=0.37). Importantly, the difference between both assays did not increase at low or high viral loads (r=0.09; p=0.24). The standard deviation was of 0.34 log_{10} copies/ml and the 95% confidence interval was ranging from -0.03 to 0.07, included in the +/- 0.50 log_{10} copies/ml limits (48). In total, 95.1% of the 185 quantified samples were inside the limits of agreement (mean +/- 1.96 SD). Four of the nine samples outside of the limits of agreement (Table 2) had a viral load inferior to the quantification threshold of 2.5 log_{10} copies/ml with the Biocentric technique, whereas only one was detected under this threshold in our assay (Table 2). Two samples, subtype H and CRF02, had higher viral load with the Biocentric technique (Table 2). However, seven samples: one subtype B, one CRF22, one CRF37, two CRF02, and two with unknown genotype had higher viral load with our new RT-qPCR method with a mean difference of -0.92 log_{10} copies/ml. Overall, our test is validated for the quantification of HIV-1 group M RNA since it has no significant difference with the reference assay.

Our panel of HIV-1 group M samples was very diverse and covered the genetic diversity of HIV-1 group M subtypes and CRFs. Each subtype or CRF was represented by one to 62 samples.
Samples for this study came from four different countries of Africa with very distinct HIV-1 circulating strains (i.e. our panel from Burundi had samples mostly from subtype C, whereas our panel from DRC harbored a high genetic diversity with 11 different subtypes or CRFs, 12 URFs and 2 from unknown genotype (Table 1)) and from one hospital in France (Table 1). To determine if the molecular epidemiological situations, i.e. different HIV-1 subtypes circulating at different frequencies, would impact negatively on our quantitative assay, we assessed the correlation between Biocentric and our technique for each studied country. We found that the Spearman correlation coefficient between the two techniques was superior to 0.95 (p<0.0001), irrespective of the country. Our test thus showed very good capacity to quantify the viral load of HIV-1 group M plasma samples, irrespective of the molecular epidemiological situation and the HIV-1 group M genotype.

Detection and quantification of HIV-1 group O strains

We tested 31 HIV-1 group O samples for viral detection with our new RT-qPCR assay, 22 were plasma samples previously tested with Abbott RealTime™ kit and nine were DNA extracts with HIV-1 group O sequence confirmation (Table 3). With our method, we detected all ten HIV-1 group O plasma samples that were previously detected with the Abbott kit, showing that our assay can readily detect HIV-1 group O viruses. We then compared viral loads of each plasma sample assessed with our technique and with Abbott RealTime™ assay. We found that our new RT-qPCR assay correlated well with Abbott test for seven out of ten HIV-1 group O samples, with a mean viral load difference of -0.05 log10 copies/ml for these seven samples (range of ∆ VL(Abbott) – VL(new qRT-PCR), -0.35 to 0.37 log10 copies/ml). We quantified three samples with a significantly higher viral load than Abbott technique (Δ, -0.72, -1.19, and -1.69 log10 copies/ml). Importantly, we could detect four additional HIV-1 group O strains that were undetected by the reference method. The viral loads of these four samples ranged from 2.18 to
3.64 log_{10}/ml (Table 3). Thus, our technique allowed us to detect and quantify more HIV-1 group O viral RNA than the reference method (higher analytical sensitivity, 64% vs 45%) and some samples detected by both methods had higher viral loads with the new method.

HIV-1 group O viruses harbor a high genetic diversity, but no subtypes have been determined such as group M classification. Here, as shown by our phylogenetic tree in the env region (Figure 1A) and also by analyses in pol region (data not shown), the strains from our panel covered the HIV-1 group O diversity. These data suggest that our technique provides a better quantification of HIV-1 group O viruses than gold-standard commercial test, irrespective of the genetic diversity.

**SIVcpzPtt and SIVcpzPts RNA detection and quantification**

The SIVcpz strains from the three infected chimpanzees were readily detected with our assay, while the two plasma samples from Ch-Ni, the SIV negative chimpanzee, were negative (Table 4), showing that our RT-qPCR assay was able to specifically detect both SIVcpzPts and SIVcpzPtt strains. Plasma samples from Cam155 were all detected and quantified and had viral loads of approximately 5 log_{10} copies/ml (Table 4). Previously, the quantification of the plasmatic SIVcpzPttCam155 RNA concentration was also carried out with the bDNA assay in 2004 (Versant HIV-1 RNA 3.0) and the Abbott RealTime™ test in 2011 (28). In 2004, the VL(Versant) was 5.09 log_{10} copies/ml, not significantly different from the viral load found with the new RT-qPCR assay. However, a viral load of 3.76 log_{10} copies/ml was found with the Abbott test in 2011, which was significantly lower than with our technique (\( \Delta \), -1.02 log_{10} copies/ml). We could detect and quantify SIVcpzPttGab2 from a plasma sample of chimpanzee Gab2 drawn in April 1988. We performed two independent RNA extractions and quantifications, and found similar viral loads around 3 log_{10} copies/ml (Table 4). The strain infecting Ch-No is from the SIVcpzPts lineage (Figure 1B), a clade more divergent from HIV-1 than SIVcpzPtt.
However, our test was still able to detect and quantify this divergent variant (Table 4). For Ch-No, we had four sequential blood samples taken between September 1989 and January 1991: the first two at the end of 1989 had an undetectable viral load and the last two in April 1990 and January 1991 had detectable viral loads of 2.68 and 3.98 log\(_{10}\) copies/ml, respectively.

Previously, Kestens and colleagues observed a fluctuating pattern with the measurement of viral titers in plasma varying from undetected to 1,000 TCID/ml (49). From the end of 1997 to 2001 (i.e. dates after our panel), Ondoa et al. quantified viral RNA from Ch-No plasma samples with a specific in-house assay and values varied from 3.93 to 5.80 log\(_{10}\) copies/ml (50, 51), in the range of our viral loads. Therefore, despite the high genetic distances between SIVcpz strains, our assay was able to detect and quantify the SIVcpz\(_{Ptt}\) and SIVcpz\(_{Pts}\) RNA from chimpanzee plasma samples.

**SIVcpz and SIVgor detection from fecal samples**

Here, we tested the detection of SIVcpz\(_{Ptt}\) and SIVgor viral RNA from fecal samples (i) to determine if our real-time RT-PCR assay was able to detect both types of viruses, direct ancestors of all HIV-1 groups, and (ii) to test whether this assay was enough sensitive for viral detection in fecal samples.

We showed previously that our test was able to detect SIVcpz\(_{Ptt}\) virus in plasma samples. Here, we tested 24 fecal samples from nine different *P. t. troglodytes* chimpanzees previously shown, by serology, to be infected with SIVcpz\(_{Ptt}\) (28, 42). Since these chimpanzees were from four different locations in south Cameroon and because of the phylogeographic clustering of SIVcpz\(_{Ptt}\), the viruses tested were expected to have high genetic distances between them (52), which could be confirmed for four of them (28, 42) (Figure 1B). Here, from a unique RNA extract, we could amplify SIVcpz\(_{Ptt}\) from only three fecal samples (corresponding to two individuals) with the conventional RT-PCR, while we could detect SIVcpz\(_{Ptt}\) from eight fecal samples.
samples (corresponding to four individuals) with the real-time RT-PCR assay (Table 5). We confirmed by sequence analyses that the amplified LTR fragments were corresponding to SIVcpzPtt (data not shown). It is thus possible to detect SIVcpzPtt in fecal samples using this real-time RT-PCR system. Although, the sensitivity of detection on this small number of samples could not be determined and further studies are needed, the sensitivity of the new assay seemed better than with conventional RT-PCR.

In addition, we tested 54 fecal samples from 22 G.g.gorilla individuals from Cameroon previously shown, by serology, to be infected with SIVgor. In previous studies (28, 42), we could amplify and sequence SIVgor small fragments with a conventional RT-PCR in only 15 samples after multiple extractions and amplification attempts (Figure 1B). Here, on a unique RNA extract, we could amplify SIVgor viruses from only four fecal samples (corresponding to three infected gorillas) with the conventional RT-PCR, while we could detect SIVgor viruses in 13 fecal samples (corresponding to eight individuals) with the real-time RT-PCR assay (Table 5). We confirmed by LTR sequencing analyses that the amplified fragments were corresponding to SIVgor. Our test is thus able to detect SIVgor viruses in fecal samples. After one attempt, the new real-time assay was able to detect SIVgor strains on 24% of the samples, compared to only 7% with the conventional method.

What is striking is that SIVcpz and SIVgor from various fecal samples from the same infected individual were not systematically detected. Thus, the main limit for SIV detection and amplification in fecal samples from great apes is not the genetic diversity of the viruses but the extremely low viral load in these samples, at the limit of detection of any PCR assay, certainly due to the sample degradation and/or the low viral shedding in feces.

Discussion
We were able to develop a new real-time RT-PCR assay with the capacity to detect and quantify a wide range of HIV-1 variants and their progenitors SIVcpz and SIVgor, infecting chimpanzees and gorillas respectively. The cost per reaction was comparable to costs of other generic or ‘in-house’ assays (lower than 15 $ for reagents and consumables) and most importantly, highly inferior to commercial tests around 50-100 $ per reaction (2).

Our assay had a high PCR efficiency with low variations between and within runs. The quantification threshold was inferior to 2.50 log_{10} copies/ml (range, 1.68 to 2.50) with an input volume of 200 µl, which is comparable with commercial and ‘in-house’ assays with the same input volume (18, 24). As shown previously, a higher plasma input volume could lower the quantification threshold our assay (18, 24), but the test was validated here on the same RNA extraction method as for the Biocentric assay to insure reproducibility and eliminate bias related to different extraction procedures. The 100% specificity of our test shows the unlikeliness of false positive results that have adverse consequences for a patient on ART with a normally undetectable viral load. Steegen and colleagues reported for example a false positive result out of 20 HIV negative samples tested with the Generic Biocentric assay (specificity, 95.0%; CI95, 73.1–99.7%) (53). The analytical sensitivity of our real-time assay, 97.4%, was calculated on 190 HIV-1 group M positive samples previously tested with the Generic Biocentric kit with a VL range of 1.68 to 7.78 log_{10} copies/ml. Only two samples with a viral load superior to the Biocentric quantification threshold were not detected with our technique, possibly because of their low viral load close to the quantification limit. However, fifteen other samples with a low to very low viral load (< 3 log_{10} copies/ml) were effectively detected by our test. Alternative explanations for false negative results would be mismatches with the primers or the probe, or a PCR inhibition. An interesting perspective would be to add an internal control to this ‘in-house’ assay to identify false negative results due to PCR inhibition; Drosten et al. showed that PCR
inhibition could concern 3.7% of reactions (45). The impact of these two undetections comparing to the low Biocentric VL results would not have major clinical consequences in resource-limited settings since with both results treated patients would not have switched their ART regimens. Actually, the threshold to determine treatment failure in resource-limited countries as recommended by the WHO in 2010 is 3.7 log_{10} copies/ml (54).

We showed that the Generic Biocentric assay and our new RT-qPCR test were highly correlated with no significant difference between their mean viral load, albeit the wide viral load range tested (1.68 to 7.78 log_{10} copies/ml), and 95.1% of quantified samples were within the limits of agreement between the two methods (47). Two samples had higher viral load with Biocentric, while seven samples from various subtypes were significantly better quantified with our method, including three that had viral loads under the Biocentric threshold. Our panel included HIV-1 group M samples from five different countries, with very diverse HIV-1 subtype/CRF distribution (19), including 39 samples from DRC and 65 from Cameroon, two countries with an extensive genetic diversity (20, 55, 56). For each country, we found an excellent correlation between both VL methods’ results, showing that HIV-1 group M diversity did not impact negatively on our viral quantification. This aspect is of major importance, and VL assays should always be validated and further evaluated in different countries with different molecular epidemiological features, as it has been done for previous ‘in-house’ assays developed for resource-limited settings (30, 45). The later criticisms on commercial viral load assays and their lack of validation on ‘unusual’ strains for developed countries (1, 16, 17, 46, 57, 58) have induced some changes in the development of industrial assays (2); i.e. the Abbott real-time assay or the last Roche Cobas taqman kit were recently validated and evaluated on HIV-1 group M diversity and few HIV-1 group O strains (18, 23, 59-62).
Unlike previously described generic or ‘in-house’ tests, the designed probe did not bear numerous mismatches with described HIV-1 group O sequences, and our RT-qPCR assay was able to detect and quantify HIV-1 group O viruses from plasma samples. Importantly, out of 22 group O samples, we were able to detect and quantify four samples that were not detected by the Abbott Real-time™ assay and we measured higher viral loads in three samples ($\bar{X}$, -1.69 to -0.72), showing that our method may be more sensitive than this commercial assay. In this study, we were able to test a high number of HIV-1 group O strains (22 plasma samples and nine DNA extracts representing 31 different HIV-1 O strains). In most studies, including the ones performed for commercial assays, only two to eleven HIV-1 group O samples were tested, which is low to assess the quality of the quantification (18, 23, 61, 62). Plantier and colleagues was the only team able to test a large number of group O samples (77 different strains) to validate their specific HIV-1 group O ‘in-house’ real-time assay (22, 26, 63). Furthermore, our group O panel covered HIV-1 group O genetic diversity, reflecting that the high genetic diversity of this group did not impact on our detection. Since other highly divergent strains (HIV-1 groups N and P) have been found in only few cases in humans, we could not test this new assay on all HIV-1 groups, but we are fairly confident that our test has this capacity since we could detect genetically distant SIVcpz and SIVgor strains.

Because of the ongoing risk of cross-species transmissions of SIVs from apes to humans (64) and the necessity to follow SIVcpz and SIVgor infection in their natural hosts to better understand the pathogenicity of these HIV-1 progenitors in their natural hosts (28, 29), our goal was to develop an assay that can detect and quantify all viruses from the HIV-1/SIVcpz/SIVgor clade. We showed here that our test is able to detect and quantify SIVcpzPtt and SIVcpzPts viruses, which are highly divergent strains, in plasma samples from western and eastern central African chimpanzees, respectively. We found that plasmatic SIVcpz viral loads in naturally
infected chimpanzees are in the range of HIV-1 viral loads in humans. The test could also detect SIVgor viruses, precursors of HIV-1 group P and probable ancestors of HIV-1 group O (27, 42, 65). The detection of SIVcpz and SIVgor RNA in fecal samples with this method was possible and more efficient than with conventional specific RT-PCR, showing this assay can be a good complement to confirm viral presence in seropositive fecal samples. However, it seems that it cannot be used for SIV screening in fecal samples to replace SIVcpz/gor serological detection because of the numerous false negative results. By this method, we confirmed that SIVcpz and SIVgor viral loads are very low in fecal samples. In general, the viral loads retrieved from infected chimpanzee plasma samples were higher than the ones obtained from fecal samples. Interestingly, SIVcpzPtt viral loads from both plasma and fecal samples could be tested for Ch-Go, and we found a more than 100-fold difference between both compartments (Tables 4 and 5, first lines). However, the sample availability was a limit here to analyze in details this pattern on a panel of associated plasma and fecal samples. The amplification of divergent variants such as SIVcpz and SIVgor was not possible with the Biocentric technique, due to numerous mismatches with the probe and the primers and we found that SIVcpz quantification was suboptimal with Abbott Real-Time™ assay. Therefore, this real-time RT-PCR test is a new opportunity to detect possible new emerging simian immunodeficiency viruses from apes to humans.

In conclusion, we developed a relatively low-cost real-time RT-PCR assay able to detect and quantify all viral strains from the HIV-1/SIVcpz/SIVgor clade, meaning that HIV-1 diversity is covered and that HIV-1 precursors can also be monitored. This new test is thus a major step in the field of viral load quantification since it could monitor any HIV-1 strains currently circulating in humans but could also detect new emergences of SIVcpz/SIVgor in humans. After this validation, an evaluation of this RT-qPCR assay on reference panels and a larger panel of
samples from a broad range of variants is needed. Also, a parallel study in a resource-limited
country would confirm its use in such settings.

Acknowledgements

We thank Fatima Mouacha for technical support. The funding of this study was supported
in part by grants from the National Institutes of Health (R01 AI50529), Agence Nationale de
Recherches sur le SIDA, France (ANRS 12182, and ANRS 12255) and the Institut de Recherche
pour le Développement (IRD). Lucie Etienne was supported by a PhD grant from Sidaction and
Fonds de dotation Pierre Bergé.
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Niphuis, P. van Eerd, G. van der Groen, P. Gigase, and et al. 1995. Phenotypic and
functional parameters of cellular immunity in a chimpanzee with a naturally acquired
simian immunodeficiency virus infection. The Journal of infectious diseases 172:957-963.
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Coppens, W. Janssens, J. Heeney, and G. van der Groen. 2001. Genetic variability of
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phylogeographic clustering of SIVcpzPtt in wild chimpanzees in Cameroon. Virology
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Democratic Republic of Congo suggests that the HIV-1 pandemic originated in Central
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RNA bDNA v3.0, Cobas AmpliPrep/Cobas TaqMan HIV-1, and NucliSens HIV-1 EasyQ
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microbiology 46:2918-2923.


Figure legends

Figure 1. Phylogenetic relationships between HIV-1 group O strains (A), and SIVcpz/SIVgor viruses (B), tested with our RT-qPCR assay with reference strains.

A - Phylogenetic tree derived from an env region (gp41; 394 bp). Grey arrows highlight strains from our panel; some strains were not sequenced because of material limitations and others were available only in pol region.

B- The tree is derived from a small env region (gp41; 248 bp), constructed by BioNJ (66). White arrows highlight strains from our fecal sample panel (except from CR4112 amplified in pol region only, and CR6278, 6466, 6495, 6534, 6682 amplified in a small 195 bp region of gp41) and black arrows highlight strains from plasma samples.

All the sequences were retrieved from the HIV database (http://www.hiv.lanl.gov/).

Figure 2. HIV-1 group M RNA viral load quantified by the Generic HIV-1 viral load Biocentric kit and our new RT-qPCR assay.

A – Samples (n=185) detected with both techniques were plotted on this linearity plot. The solid line represents the fitted regression. Pearson correlation r, 0.95 (p<0.0001). The 2.50 log10 copies/ml limit of detection is represented by grey dashed lines.

B - The 185 samples detected with both techniques were plotted on this Bland-Altman difference plot. In vertical axis, the difference between Biocentric and new RT-qPCR assay viral load, against the mean viral load between the two techniques, in horizontal axis. The mean bias on the difference (solid line, $\hat{d}=0.023 \log_{10}$ copies/ml) and limits of agreement (dashed lines) are shown on the graphic. On the right vertical axis are represented two main limits: the +/- 0.5 log10 copies/ml limit and the CI95 interval (-0.027 to 0.072 log10 copies/ml).
Table 1. Selected HIV-1 group M plasma samples tested with the new RT-qPCR assay.

DRC, Democratic Republic of Congo. a, The total line includes the 23/190 samples with unknown genotypes.

<table>
<thead>
<tr>
<th>Subtype/CRF</th>
<th>Burundi</th>
<th>Cameroon</th>
<th>DRC</th>
<th>Togo</th>
<th>France</th>
<th>Total</th>
</tr>
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<tbody>
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<td>A</td>
<td>2</td>
<td>-</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td>B</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>14</td>
<td>14</td>
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<td>C</td>
<td>16</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
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<td>G</td>
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<td>-</td>
<td>2</td>
<td>3</td>
<td>-</td>
<td>5</td>
</tr>
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<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>J</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
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<td>12</td>
<td>6</td>
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<tr>
<td><strong>Total a</strong></td>
<td><strong>18</strong></td>
<td><strong>65</strong></td>
<td><strong>39</strong></td>
<td><strong>54</strong></td>
<td><strong>14</strong></td>
<td><strong>190</strong></td>
</tr>
</tbody>
</table>
Table 2. HIV-1 group M samples out of the limits of agreement between our new RT-qPCR method and the Generic Biocentric assay.

Seven samples, out of the limits of agreement (mean +/- 1.96 SD), had a higher viral load with our quantification assay comparing to the Generic Biocentric test (top panel) and two had a lower viral load (bottom panel). For each sample, the country of origin and the genotype are given with the viral loads (VL in log_{10} copies/ml) obtained from both techniques and the difference between them ($\delta = \text{VL(Biocentric)} - \text{VL(new RT-qPCR)}$). Asterisk, viral load inferior to the 2.5 log_{10} copies/ml threshold.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Country</th>
<th>Genotype</th>
<th>VL(Biocentric)</th>
<th>VL(new qRT-PCR)</th>
<th>$\delta$</th>
</tr>
</thead>
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<tr>
<td>3355</td>
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<td>CRF02</td>
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<td>6.78</td>
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<tr>
<td>625</td>
<td>DRC</td>
<td>CRF02</td>
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<td>5004</td>
<td>Togo</td>
<td>-</td>
<td>1.68*</td>
<td>2.59</td>
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<tr>
<td>596</td>
<td>DRC</td>
<td>CRF37</td>
<td>3.23</td>
<td>4.13</td>
<td>-0.81</td>
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<td>3023</td>
<td>Cameroon</td>
<td>CRF22</td>
<td>2.34*</td>
<td>3.13</td>
<td>-0.79</td>
</tr>
<tr>
<td>24</td>
<td>France</td>
<td>B</td>
<td>1.81*</td>
<td>2.45*</td>
<td>-0.64</td>
</tr>
<tr>
<td>543</td>
<td>DRC</td>
<td>H</td>
<td>3.69</td>
<td>2.95</td>
<td>0.74</td>
</tr>
<tr>
<td>5053</td>
<td>Togo</td>
<td>CRF02</td>
<td>6.83</td>
<td>6.01</td>
<td>0.82</td>
</tr>
</tbody>
</table>
Table 3. HIV-1 group O detection and quantification by RT-qPCR.

The table is divided in two main parts: the top part for plasma samples, to test HIV-1 group O detection and quantification; the lower part for DNA samples, to test for HIV-1 group O detection. Sample identifications (ID) are given. For each sample, the viral loads (VL in log_{10} copies/ml) obtained from both techniques are given if it could be detected (the negative signs in VL columns reflect undetection of samples) and the difference between them is calculated (\( \delta = \text{VL} \) (Abbott) – \text{VL} (new RT-qPCR) ; in log_{10} copies/ml). ‘New + / Abbott –’ shows that only the new assay could detect and quantify the corresponding strains.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>VL (Real Time Abbott)</th>
<th>VL (new qRT-PCR)</th>
<th>( \delta )</th>
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<tbody>
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<td>Plasma samples for quantification</td>
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<tr>
<td>YD1396</td>
<td>2.28</td>
<td>2.43</td>
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<tr>
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<td>0.37</td>
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<td>YD1431</td>
<td>2.53</td>
<td>4.22</td>
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<td>CM2080</td>
<td>3.04</td>
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<td>03/096/A66</td>
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<td>YD656</td>
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<td>3.70</td>
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<td>SKPI1015</td>
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Table 4. SIVcpzPtt and SIVcpzPts detection and quantification by RT-qPCR in plasma samples.

Two to four plasma samples per individual from sequential blood tests from SIVcpz infected chimpanzees (Ch-Go, Gab2 and Ch-No) as well as a SIV negative chimpanzee (negative control, Ch-Ni) were tested for the presence of SIV antibodies (Serology), and detection and quantification of viral RNA by the new RT-qPCR assay. a, RNA extraction performed in Cameroon and quantification performed in Montpellier; b, duplicates; c, viral titer in the plasma, 100 TCID/ml (Tissue culture infected dose) (49).

<table>
<thead>
<tr>
<th>Chimpanzee ID subspecies</th>
<th>SIVcpz</th>
<th>Plasma Sample ID</th>
<th>RNA extraction date</th>
<th>Serology</th>
<th>Detection</th>
<th>Quantification (log_{10} copies/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ch-Go P. t. troglodytes</td>
<td>SIVcpzPtt-Cam155</td>
<td>CAM155-01.05.04</td>
<td>26.02.09</td>
<td>+</td>
<td>+</td>
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<td></td>
<td></td>
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<td>01.04.11^a</td>
<td>+</td>
<td>+</td>
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Table 5. SIVcpzPtt and SIVgor detection from fecal samples with our real-time RT-PCR assay.

Fecal samples from chimpanzees (panel A) and gorillas (panel B) from Cameroon, positive in the HIV-1/-2 Innolia™ serological assay, were subjected to our new real-time RT-PCR test. For each sample tested the sample number is given with the ID of the corresponding individual (e.g. CPg-ID1, abbreviation of the collection site followed by a letter for the species (g, gorilla; c, chimpanzee). a, some samples were previously confirmed SIV positive by conventional RT-PCR after multiple extraction and amplification attempts (result of two to ten amplification attempts from one to ten different RNA extracts). For each sample and for a given RNA extract, we performed a conventional RT-PCR in gp41 region (b) and a real-time RT-PCR with our new protocol (c); for information (d), values obtained with the real-time RT-PCR ranged between 1.63 and 2.84 log_{10} copies/ml. Pos, positive; -, negative.
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